LPAR2 Regulates LPA-Induced Osteoclast Sealing Zone Dynamics

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LPAR2 REGULATES LPA-INDUCED OSTEOCLAST SEALING ZONE DYNAMICS

Stephanie M. Meyer

Abstract

Bone metastasis is an excruciating consequence of multiple primary cancers, and is commonly treated with bisphosphonate drugs. Osteoclasts, bone-resorbing cells vital to proper bone remodeling and fracture healing, are responsible for the formation of osteolytic bone tumors. Osteoclasts function through an actin-based structure called the actin sealing zone, or actin ring. Actin ring formation is required for proper bone resorption, and can be used to measure osteoclast function.

The Lee lab is investigating the mechanisms of lysophosphatidic acid (LPA) treatment on osteoclasts. LPA is a lipid molecule found at elevated levels in the blood of cancer patients. In preliminary *in vitro* experiments, osteoclasts were directly treated with LPA. Cells were also exposed to LPA in the presence of a common bisphosphonate, Zometa, which is currently used to treat the osteoporosis commonly observed in cancer patients. In the absence of Zometa, LPA increases the actin ring circumference and the number of cellular nuclei. Zometa treatment decreases the actin ring perimeter and number of nuclei. Osteoclasts treated with Zometa and LPA simultaneously show an expanded actin ring and elevated number of nuclei, similar to the phenotype induced by exposure to LPA alone. Cells treated with Zometa after LPA exposure showed no observable response to the drug.

LPA works through five known receptors, three of which are defined in osteoclasts. The LPA receptor (LPAR) 1/3 inhibitor Ki16425 did not suppress the actin ring increase by LPA. However, LPAR2 agonist FAP12 demonstrated effects identical to those observed in the presence of LPA on bone. Such data suggests that LPAR2 is responsible for the actin ring

perimeter and nuclear quantity changes seen with LPA treatment. Small interfering RNAs (siRNAs) specific to LPAR2 were utilized to examine LPAR2 function. Knockdown of LPAR2 inhibited the actin ring expansion observed in cells with functional LPAR2. The results suggest that LPAR2 is a key element in LPA-induced actin sealing zone dynamics, and is consequently a new target for alternative drug therapies to treat LPA-induced bone metastasis.

1. Introduction

Bone remodeling occurs continuously throughout an individual's lifetime, and it is critical to bone growth, fracture healing, and homeostasis of blood calcium and phosphate levels. Approximately 10% of the bone mass of an adult human is replaced annually, equating to turnover of the entire skeleton in the span of one decade (Alliston 2002). During bone remodeling osteoclasts degrade bone, while osteoblasts replenish the degenerate bone structure. Pathologies such as osteoporosis (deficient bone mass) and osteopetrosis (abnormally dense bone mass) result if the activity of both cell types is not in equilibrium (McMichael 2008). Osteoclasts are multinucleated cells, measuring up to 100 microns in diameter and containing an average of 4 to 20 nuclei within the cell body (McMichael 2008). RANKL (Receptor Activator for Nuclear Factor κ B Ligand) is secreted by osteoblasts in the bone environment, and is mainly responsible for stimulating osteoclast differentiation through the activation of multiple signaling cascades (Teitelbaum 2007). Critical to the resorption function, osteoclasts form F-actin ring-like structures, referred to as "sealing zones" (Teitelbaum 2007).

Formation of an F-actin sealing zone, or actin ring, is critical to the bone resorption process (Saltel 2004). The resorption process can be divided into three stages: adhesion of the osteoclasts to the bone surface, resorption of the bone matrix, and migration of the osteoclast (Saltel 2004). The actin sealing zone anchors the osteoclast to the bone surface via integrins, most notably α_vß3 (Saltel 2004). Other labs have concluded that osteoclasts "can only resorb mineralized matrices," as resorption has not been seen to occur on demineralized bone (Saltel 2004). After the osteoclast is anchored to the bone surface, the cell becomes polarized and forms an "actin patch" structure, which is thought to develop into the actin sealing zone (Lee 1999). A signaling cascade releases proteolytic substances, such as hydrochloric acid and

Cathepsin K (CatK), into the bone microstructure (Zou 2010). Following resorption, the osteoclast "spreads," describing a depolarized state in which the cell does not have an actin ring structure (Saltel 2004). During this spreading state, the osteoclast migrates to a new location on the bone surface, creating a resorption pattern thought to be determined by certain glycoproteins on the bone surface (Saltel 2004). A new actin ring is formed as the cell polarizes, repeating the cycle as indicated in **Figure 1a**.

Bone is the most common site of secondary metastasis in breast cancer patients, suggesting that the biochemical ramifications of cancer may affect the cells responsible for maintenance of the bone matrix (Pavlakis 2006). Previous studies have implicated lysophosphatidic acid (LPA, **Figure 1d**) as a key signaling molecule involved in cancer cell proliferation. LPA is a phospholipid signaling molecule that is speculated to incite the secondary metastasis of breast cancer to bone (Boucharaba 2006). For example, elevated LPA levels have been observed in the blood of breast cancer patients (Nogouchi 2009). Thus far, five LPA cell receptors have been characterized, and at least three are present in osteoclasts (Nogouchi 2009, Lapierre 2008). While research has been done to examine the LPA-induced activation of breast cancer cells, as well as the inhibition of cancer cell apoptosis by LPA, the effect of aberrantly high LPA levels on osteoclasts has not been well-characterized (Nogouchi 2009).

Since bone metastasis accounts for thirty to forty percent of all recurrences of primary breast cancer, several therapeutic approaches have been employed to prevent the spread of malignant cells to secondary sites throughout the body (Suva 2010). One clinical treatment is the use of bisphosphonate drugs, such as Zometa (zoledronic acid). Zometa is a common bisphosphonate prescribed to prevent bone complications following primary cancers (Gnant 2009). Bisphosphonate drugs inhibit bone matrix degradation via induction of osteoclast

apoptosis, or programmed cell death (**Figure 1c**) (Beauchamp 2002). Bisphosphonates are also currently used to treat non-cancerous bone abnormalities. Examples include pediatric *osteogenesis imperfecta*, a condition in which osteoblasts do not produce sufficient bone matrix to replace the minerals resorbed by osteoclasts, and *osteoporosis*, in which osteoclasts resorb more bone matrix than is replaced by the osteoblasts (Lindsay 2002). One study suggests that bisphosphonates biochemically inhibit LPA from inducing migration of human ovarian cancer cells (Sawada 2002). Another research group has suggested that Zometa has anti-cancer progression effects (Gnant 2009). However, while such studies assume bisphosphonates' ability to simply inactivate the osteoclast, the effect of LPA on bisphosphonates' activity in the context of osteoclast bone resorption remains uninvestigated (Gnant 2009). The following project examines the effects of LPA on osteoclasts, as well as LPA's effect on the interactions of bisphosphonates with the osteoclasts themselves.

2. Methods

2.1 *Preparation of Osteoclasts*

Primary cells were generated by extracting monocytes from mouse bone marrow, maturing them into macrophages with M-CSF (Macrophage Colony Stimulating Factor), and differentiating the cells into adult osteoclasts upon addition of RANKL. RANKL, or Receptor Activator for Nuclear Factor κ B Ligand, is a molecule naturally present on osteoblasts that incites activation of osteoclasts. Thin slices of ivory were stored in PBS (phosphate-buffered saline), soaked in cell culture media, and placed into the wells of a sterile plate. Mature macrophages were quantified and diluted with PBS to get the recommended concentration of cells (approximately 1 x 10^5 cells /6-well plate, detected using hemacytometry). Dulbecco's Modified Eagle Medium (DMEM, 3 mL) and RANKL (2 μL) were also added to each well, and cultures were incubated for 48 hours at 37°C (Krits 2002)

2.2 *Fixation of Osteoclasts*

After cells were treated under various conditions (addition of LPA, Zometa, Ki16425 inhibitor, FAP12), the cultures were quickly fixed in 1% formaldehyde and then additionally fixed in 2% formaldehyde. Cells were stained using standard methods for either Phalloidin or LPAR2-specific primary antibodies followed by fluorescent secondary antibodies. The slides were viewed under the Zeiss Meta Confocal microscope or a Nikon Epifluorescence microscope, and the actin sealing zone perimeters were measured using Sigma Scan Pro 5.0. Averages and standard deviations were calculated and compared (Zeng 2000).

2.3 *Bone Assays*

Differentiated RAW 264.7 osteoclasts were plated on BD-osteological discs or ivory for three days, while being treated with either vehicle control or LPA/FAP12. Cells were removed and viewed under low magnification with the Nikon epifluorescence microscope. For MBM primary assays, mature osteoclasts were plated on ivory and treated for three days with vehicle or LPA/FAP12. Cells were removed, and the remaining pits were stained with acid hematoxylin to be viewed with the Zeiss Meta Confocal microscope.

2.4 *siRNA Suppression of LPAR2 Gene*

siRNAs (75071, 74987, 74894) were purchased from Ambion and transfected using Lipofectamine 2000 from Invitrogen. Cells were differentiated four days before control or LPAR2-specific siRNAs were added to Lipofectamine 2000. For bone assays, immediately following the transfection, cells were scraped and re-plated on ivory. RNA or protein was harvested two days post-transfection. Competitive RT-PCR was utilized to analyze the decrease in RNA. An internal standard was created that contained the same primer-binding sequence as LPAR2, but lacked approximately 20% of the internal sequence. 300 fg of internal standard was added to 1 μg of sample prior to reverse transcriptase, and then subject to standard PCR conditions. Western analysis was used to analyze protein following standard lab protocols (Zeng 2000).

2.5 *Reagents*

Lysophosphatidic acid (LPA) was used at a 2 μM concentration and ordered from the Cayman Chemical Company. Zometa (zoledronic acid), provided by Dr. Thomas Rosol of The

Ohio State University College of Veterinary Medicine, was used at a concentration of 3.75 ng/mL. Ki16425 inhibitor was used at 10 μM, and obtained from the Cayman Chemical Company. FAP12, ordered from BioMol, was used at a concentration of 50μM. Control and LPAR2-specific siRNAs (75071, 74987, 74894) were acquired from Ambion and were used at a 75 nM concentration, and rabbit polyclonal LPAR2-specific antibodies were ordered from Abcam. Bisbenzamide was purchased from Sigma Aldrich, and both Phalloidin and Lipofectamine 2000 were purchased from Invitrogen.

3. Results

The Effects of LPA and Zometa on Actin Sealing Zone Perimeter and Nuclear Number

A search through previous data indicates that there are currently no published papers in which LPA was directly added to osteoclasts. I began my studies by assessing the initial changes seen in osteoclasts by the addition of LPA alone and LPA in the presence of Zometa.

3.1 *LPA Increases Osteoclast Sealing Zone Perimeter, Nuclear Number and Resorption*

Osteoclasts derived from both an immortalized cell line (RAW 264.7 cells), as well as fresh cells derived from mouse bone marrow (MBM cells) were plated in wells that contained four to six bone slices. LPA (2 μM) was added to one well, and the other well was left untreated as the vehicle control. The cultures were incubated at 37°C for 24 hours, and then the bone slices were fixed and stained with fluorescent Phalloidin and bisbenzamide to view the actin sealing structures and nuclei.

Data indicates that LPA increases sealing zone perimeter, relative to osteoclasts unexposed to LPA **(Figure 2a)**. Both RAW 264.7 cells and MBM primary cells exhibited sealing zones with perimeters approximately double the size of the control **(Figure 2b)**. Also, nuclear number increased in both subsets of cells following LPA treatment **(Figure 2c)**. Larger osteoclasts, deemed as such by a greater number of nuclei, are more likely to be in an active state of resorption (Lees 2001). This data indicates that LPA has a direct effect on osteoclasts.

Differentiated osteoclasts were also plated on either BD-osteological discs or ivory. RAW 264.7 cells were treated with either vehicle solution alone or LPA, and cultured on osteological discs at 37°C for three days. Cells were then removed and the clearings quantified. Additionally, MBM primary cells were subject to the same conditions, on ivory medium. After

removing cells from the culture surfaces and staining resorbed zones with hematoxylin, the pits were quantitatively characterized. On the synthetic osteological disks, LPA increased the resorption area and the total percentage of the bone surface resorbed, relative to a 100% resorption control (**Figure 2d**). LPA did not significantly affect the number of resorption pits formed. The MBM primary cells cultured on bone showed that LPA increased the areas of the individual resorption pits relative to the untreated control cell resorption zones; however, no change in resorption pit height was seen (**Figure 2d**).

3.2 *LPA and Zometa have Contrary Effects*

Osteoclasts were cultured on bone, as described in the "Preparation of Osteoclasts" protocol. LPA was added to one well, Zometa was added to the second well, and both LPA and Zometa were added to the third well. The cells were incubated at 37°C for 48 hours, and then fixed and stained with fluorescent Phalloidin to view the actin sealing zone. The cells were viewed using confocal microscopy and the actin rings measured. The nuclei of cells with actin rings were also stained with bisbenzamide and quantified.

Figure 3a shows that while LPA increased the perimeter of the actin sealing zone, Zometa decreased the sealing zone perimeter. However, when osteoclasts were exposed to both LPA and Zometa simultaneously, the sealing zone perimeter increased, measuring comparably to the sealing zone size of osteoclasts exposed only to LPA (**Figure 3b)**. The nuclear number data revealed a similar pattern (**Figure 3c**). Such data implies that LPA may interfere with the mechanism of how bisphosphonates, particularly Zometa, downregulate osteoclast resorption.

3.3 *Pre-treatment with LPA Makes Zometa Ineffective*

To determine whether the chronological order of LPA and Zometa addition had any effect on the actin sealing zone or nuclei phenotypes, osteoclasts were cultured on bone for four days. LPA or Zometa was added to each well and incubated at 37°C for 24 hours. After 24 hours, LPA or Zometa was added so that one sample had two days of Zometa treatment and one day of LPA exposure (2D Zometa-1D LPA), and the other sample had two days of LPA exposure and one day of Zometa treatment (2D LPA-1D Zometa). After a total incubation time of 48 hours, slides of the osteoclasts were fixed and stained with Phalloidin and bisbenzamide to view the osteoclasts' actin rings. Actin rings and nuclei were quantified.

Exploration of the time effects of LPA and Zometa on osteoclasts revealed that when osteoclasts are treated with Zometa 24 hours prior to LPA exposure, the measurable actin sealing zones remained small, similar to untreated control cells. However, the majority of cells did not have measurable actin rings after 24 hours of treatment with Zometa, as depicted in **Figure 4a**. However, if cells are subjected to LPA 24 hours before exposure to Zometa, the actin ring size increases as in osteoclasts exposed to LPA alone (**Figure 4b**). The nuclear number data trended with the actin ring data (**Figure 4c**). This data reveals that if a high level of LPA is present before Zometa is administered, then the effects of Zometa on actin sealing zone formation may be negated.

The Molecular Mechanism of LPA Action

Since LPA has a direct effect on osteoclasts and can override Zometa treatment, I continued my studies to better understand how LPA regulates osteoclasts. There are currently five known LPA receptors, and three are known to be present in osteoclasts. The following

assays were designed to elucidate the particular receptor through which LPA functions, as well as to determine the specific effects that result from activation of the receptor.

3.4 *LPA is Not Inhibited by LPAR1/3 Antagonist Ki16425*

Osteoclasts were cultured on bone and treated with either vehicle, LPA, or with Ki16425 inhibitor in the presence of LPA. Ki16425 is a pharmacological inhibitor that acts specifically blocks LPAR1 and LPAR3. The cells were incubated for 24 hours, and stained with Phalloidin and bisbenzamide according to standard lab protocol. The actin sealing zone sizes were measured and the nuclear number counted.

Even with LPAR1 and LPAR3 biochemically hindered by the Ki inhibitor, the sealing zone perimeter still increased (**Figures 5a, b).** Ki16425 inhibitor was added at 2x and 10x concentration and still had no effect on actin ring expansion or nuclear number (data not shown). This result suggests that LPAR1 and LPAR3 are not major receptors involved in LPA's mechanism for increasing the actin sealing zone perimeter. The number of nuclei also increased when treated with LPA and Ki16425 inhibitor, mimicking the nuclear effects observed with LPA alone (**Figure 5c**). Additionally, Ki16425 inhibitor was also added to cells exposed to LPA for two days and Zometa for one day (2D LPA- 1D Zom) as described in **Results 2.3** (data not shown**)**. While doing so slightly decreased actin ring perimeter and nuclear number, the reduction of both osteoclast structures did not reach near normal levels. This data implies that even with suppression of LPAR1 and LPAR3, LPA still interacts with the osteoclast in order to increase the sealing zone and nuclear number.

3.5 *LPAR2 Agonist FAP12 Mimics LPA Effects*

Osteoclasts were treated with either LPA or FAP12. FAP12 is an LPA agonist that acts specifically to simulate activation of LPAR2 by LPA. The cells were incubated for 24 hours, and stained with Phalloidin and bisbenzamide to measure the actin sealing zone sizes and nuclear number.

When LPA agonist FAP12 was added to osteoclasts, the same degree of actin sealing zone expansion resulted as when cells were treated with LPA alone (**Figures 6a, b**). Nuclear number data shows that there is also an increase in cell fusion when osteoclasts were treated with FAP12, as seen in the osteoclasts treated with LPA (**Figure 6c**). Since FAP12 interacts specifically with LPAR2 and no other known LPA receptors, such data suggests that LPA's interaction with LPAR2 is primarily responsible for the larger perimeter of the osteoclast's actin sealing zone.

MBM-derived osteoclasts were plated on ivory and either left untreated in vehicle solution, were treated with LPA, or were treated with FAP12. The cells were incubated for 72 hours at 37°C, removed from the ivory, and stained with acid hematoxylin to be viewed using epifluorescent microscopy. Both the LPA and FAP12 treated cells exhibited an increased resorption pit area relative to the untreated control cells. However, the height of the resorption pits remained statistically similar upon comparison of LPA/FAP12 treated cells versus the untreated control (**Figure 6d**).

3.6. *siRNA Suppression of the LPAR2 Gene Diminishes LPA Effects*

Osteoclasts were stained with LPAR2-specific antibodies. LPAR2 was found throughout the osteoclast, indicating that the LPAR2 protein's location is not specific to the actin structures

(Figure 7a). Three LPAR2-specific siRNAs were used to inhibit expression of the LPAR2 gene. Controls of LPAR2 siRNAs were transfected via lipid-based Lipofectamine 2000 on day four of osteoclast differentiation, and RNA (**Figure 7b**) or protein (**Figure 7c**) was harvested and assessed two days post-transfection. A competitive RT-PCR confirmed that all three siRNAs inhibited the sample RNA expression, as the top band in **Figure 7b** represents the sample LPAR2 RNA and the bottom band is the internal standard control. Western blot analysis showed that siRNA1 and siRNA2 inhibited protein expression most effectively, while the ß-actin control remained constant (**Figure 7c**).

To further test if LPAR2 is the primary LPA receptor responsible for the LPA-induced actin ring changes, osteoclasts on ivory were transfected with siRNAs for either a nonsense control (C) or LPAR2 (Si) in both the presence and absence of LPA. The cellular actin was stained with Phalloidin to view sealing zone formation. Knock-down of LPAR2 resulted in smaller actin sealing zones relative to the control in the presence of LPA (**Figure 8b**). Also, as indicated in the graph in **Figure 8a**, an increase in the actin ring size only occurred when a cell containing control siRNA, non-specific to LPAR2, was treated with LPA. Since there was no significant increase in actin sealing zone size in cells containing the LPAR2-specific siRNA, this data confirms that the LPAR2 protein is a major receptor in osteoclasts for regulating sealing zone expansion.

4. **Discussion**

The data from the experiments provides three major conclusions. First, LPA causes expansion of the actin sealing zone, as well as an increase in the number of nuclei in the cell body. Second, not only does LPA cause an increase in sealing zone size, but the data suggests that LPA has a mechanism that negates activity of bisphosphonate drugs, specifically Zometa. Third, after inhibiting LPAR1 and LPAR3 with Ki16425 inhibitor, as well as using FAP12 to stimulate only LPAR2 and confirming the effect through LPAR-2 specific siRNA suppression, it is likely that LPAR2 is the primary osteoclast receptor responsible for the LPA-induced phenotypes of the actin sealing zone and nuclear number.

This is the first documented study that looks at the direct effects of LPA on mature osteoclasts, and the findings of this project create new avenues for both clinical and benchtop exploration. Knowledge of such cellular effects may have substantial implications in the clinical setting. Understanding the mechanisms of LPA action may lead to determining whether or not a correlation exists between threshold levels of serum LPA and those breast cancer patients who experience secondary bone metastasis. Also, if LPA levels become increasingly elevated as cancer progresses in patients, then prescribing Zometa to late-stage cancer patients may not be as effective as physicians are expecting. Such information could improve treatment guidelines for breast cancer patients. Zometa may be administered in early stages of the cancer, and perhaps preventative doses of bisphosphonate could be prescribed to those deemed high risk for developing the cancer.

Other areas of research to pursue in this project involve looking at the different forms of LPA that may interact with osteoclasts. In this project, 1-oleoyl lysophosphatidic acid was used, mainly because it is the most potent LPA analog characterized, and stimulates all five known

LPA receptors (van Corven 1992). Cayman Chemicals Company manufactures a variety of LPA molecules with different side chains. These modified versions of LPA include OMPT, 1-octadectyl lysophosphatidic acid, and other varieties that only stimulate specific LPA receptors. Different cancer cell types may produce different forms of LPA, and examining the effect of these different LPA analogs on bone would also provide insight into the mechanism by which the primary cancer metastasizes to bone.

The knowledge that LPAR2 is a major receptor involved in LPA-mediated actin sealing zone formation also has substantial implications. Pharmaceutical isolation of pathways that are responsible for this increase in LPA levels may pioneer a new direction of benchtop research that has the potential to decrease the formation of secondary bone tumors. For example, autotaxin is a protein induced by cancer cells that stimulates LPA production by circulating platelets in the blood (David 2010). Perhaps rather than targeting the osteoclasts themselves, hindering autotaxin's ability to induce these elevated LPA levels is a more effective means of preventing formation of the actin sealing zones that are necessary to osteoclast activity. Interfering with the LPA-LPAR2 interaction prior to the actin sealing zone (and consequent resorption) increase may prove more practical than simply treating patients with a bisphosphonate (**Figure 2d**).

Other labs have associated LPA with anti-apoptotic activity in leukemia cells (Kumar SA). In one published case study, a cancer-free patient who had been treated long-term with bisphosphonates presented with giant osteoclasts that exhibited decreased resorptive function and a resistance to phagocytosis (Jain 2009). Such results consequently increased the number of osteoclasts, which contrast my results that show the cells unable to form an actin ring when exposed first to Zometa and then LPA (**Figure 4a**). These findings challenge researchers to further explore the mechanisms by which bisphosphonate activity is affected by LPA. Such

research, in conjunction with my demonstration that LPA's demonstrated anti-apoptotic mechanism overrides Zometa's apoptotic activity, implies that clinicians need to treat LPA up-regulation along with the cancer itself.

The siRNA knock-down of the LPAR2 gene also clarifies an important point in the understanding of how LPA affects the actin sealing zone. As seen in **Figure 8b**, suppression of LPAR2 protein in the presence of LPA results in smaller actin sealing zones than those with a fully functional LPAR2 gene product. However, osteoclasts with a knocked-down LPAR2 gene still *formed* an actin sealing zone, implying that although LPAR2 is important in sealing zone expansion, it is not solely responsible for the formation of the actin structure. **Figure 7a** shows images in which osteoclasts were stained with an LPAR2-specific antibody (red), illustrating that LPAR2 is not present in the actin sealing zone itself (green), and LPAR2 is consequently not functioning to form the sealing zone. I propose that there is an external signaling mechanism involved in this ring expansion, induced by LPA.

The siRNA knock-down data strongly suggests that LPAR2 is primarily responsible for the expansion of the actin sealing zone. However, the roles of LPAR1 and LPAR3 should not be ignored. For example, LPAR1 was found to be the predominantly expressed LPA receptor in osteoclasts, and is speculated to be the receptor through which osteoblast-produced LPA reduces apoptosis of osteoclasts, extending the cell life (Lapierre 2008). LPAR3 and other LPA receptors not yet characterized in osteoclasts may still be involved in the formation and turnover of actin structures. This study focused on LPAR1, LPAR2, and LPAR3 because my lab had access to a pharmacological antagonist (Ki16425) and agonist (FAP12) for these receptors. When the FAP12 agonist cell treatment produced identical results to those generated with LPA treatment, experiments focused on the molecular aspects of LPAR2 expression. That being said,

the potential impact of other LPA receptors cannot be overlooked, and requires further investigation.

The results proving that adding LPA to cells increases the nuclear number leads to another area of conjecture. One may speculate that the increased number of nuclei results from the LPA-induced fusion of cells. From previous research conducted in the Lee lab, a correlation between actin sealing zone perimeter and nuclear number was established (McMichael 2009). Using this data, the actin sealing zone increase that was observed in my experiments fell in the normal ranges for the number of nuclei that were observed. The mechanism by which LPA increases the degree of osteoclast fusion is uncertain. The higher nuclear number may be a result of the LPA physiologically improving the osteoclasts' ability to fuse, or the LPA-induced consequence of a decreased rate of apoptosis that increases the cells' relative proximity and probability of fusing. There may also be a motility effect that is enhanced by the LPA's interaction with the cell, bringing the osteoclasts closer together. The ramifications of LPA increasing the nuclear number and correlated bone resorptive capacity emphasize the importance of understanding the interaction of LPA with osteoclasts.

5. References

Alliston T, Derynck R. (2002) "Medicine: interfering with bone remodeling." *Nature.* 416 (6882): 686-87.

Beauchamp, K. (2002, Published under Lindsay, R). "Modeling the Benefits of Pamidronate in Children with Osteogenesis Imperfecta." *Journal of Clinical Investigation*. 2002; 110(9): 1239-41. Doi:10.1172/JCI17051.

Boucharaba A, Serre CM, Guglielmi J, Bordet JC, Clézardin P, Peyruchaud O. (2006) "The Type 1 Lysophosphatidic Acid Receptor is a Target for therapy in Bone Metastases." *The Proceedings of the National Academy of Sciences, USA*. 103(25): 9643-8. Epub 2006 Jun 12.

David M, et al. (2010) "Cancer Cell Expression of Autotaxin Controls Bone Metastasis Formation in Mouse through Lysophosphatidic Acid-dependent Activation of Osteoclasts." *PLOS One.* 5(3): e9741.

Gnant M, et al. (2009) "Endocrine Therapy plus Zoledronic Acid in Premenopausal Breast Cancer." *New England Journal of Medicine.* 360(7):679-91. Erratum in: N Engl J Med. 360(22):2379.

Jain N, Weinstein R. (2009) "Giant Osteoclasts After Long-Term Bisphosphonate Therapy: Diagnostic Challenges." *Nature Reviews: Rheumatology.* 5(6): 341-6. Doi: 10.1038/nrrheum.2009.87.

Krits I, Wyslomerski RB, Holliday LS, Lee BS. (2002) "Differential Localization of Lyosin II Isoforms in Resting and Activated Osteoclasts." *Calcified Tissue International*. 71: 530-38.

Kumar SA, Hu X, Brown M, Kuschak B, Hernandez TA, Johnston JB, Gibson SB. (2009) "Lysophosphatidic Acid Receptor Expression in Chronic Lymphocytic Leukemia Leads to Cell Survival Mediated though Vascular Endothelial Growth Factor Expression." *Leukemia and Lymphoma*. 50(12): 2038-48.

Lapierre DM, Leblanc MM, Pereverzev A, Dixon SJ, Sims SM. (2008) "Lysophosphatidic Acid Regulates Osteoclast Survival and Retraction." *Journal of Bone and Mineral Research*. 23(1): 5268.

Lee BS, Gluck SL, Holliday LS. (1999) "Interaction Between Vacuolar H+-ATPase and Microfilaments During Osteoclast Activation." *Journal of Biological Chemistry*. 274(41): 29164-71.

Lees RL, Sabharwal VK, Heersche JN. (2001) "Resorptive state and cell size influence intracellular pH regulation in rabbit osteoclasts cultured on collagen-hydroxyapatite films." *Bone.* 28:187-194.

Lin M, Herr D, Chu J. (2010) "Lysophosphatidic acid (LPA) Receptors: Signaling Properties and Disease Relevance." *Prostaglandins and Other Lipid Mediators*. 91 (3,4): 130-38.

Lindsay, R. (2002) "Modeling the Benefits of Pamidronate in Children with Osteogenesis Imperfecta." *Journal of Clinical Investigation*. 110(9): 1239-41. Doi:10.1172/JCI17051.

Manolson, MF, Yu H, Chen W, Yao Y, Li K, Lees R, Heersche JN. (2003) "The a3 Isoform of the 100 kDa V-ATPase Subunit Is Highly but Differentially Expressed in Large ($\geq$ 10 Nuclei) and Small ($\leq$ 5 Nuclei) Osteoclasts." *Journal of Biological Chemistry*. 278(49): 49271-78.

McMichael BK. (2008) "Myosin IIA Regulates Cell Fusion in Osteoclasts." *The Ohio State University Knowledge Bank.* <https://kb.osu.edu>. 2008 April Issue.

McMichael BK. (2009) "Regulated Proteolysis of Nonmuscle Myosin IIA Stimulates Osteoclast Fusion." *The Journal of Biological Chemistry*. 284(18): 12266 -12275.

Nogouchi K, Herr D, Mutoh T, Chun J. (2009) "Lysophosphatidic Acid and Its Receptors." *Current Opinions in Pharmacology.* 9(1): 15-23.

Pavlakis N, Schmidt R, Stockler M. (2006) "Bisphosphonates for Breast Cancer." *ACP J Club*. 144(2):39.

Saltel F, Destaing O, Bard F, Eichert D, Jurdic P. (2004) "Apatite-Mediated Actin Dynamics in Resorbing Osteoclasts." *Molecular Biology of the Cell*. 15(1): 5231-41.

Sawada K, Morishige K, Tahara M, Kawagishi R, Ikebuchi Y, Tasaka K, Murata Y. (2002) "Alendronate Inhibits Lysophosphatidic Acid-Induced Migration of Human Ovarian Cancer Cells by Attenuating the Activation of Rho." *Cancer Research.* 1: 62(21): 6015-20.

Suva LJ. (2010) "Adjuvant Bisphosphonates in Breast Cancer: The ABCSG-12 Study." *Current Osteoporosis Reports*. 8(2):57-59.

Teitelbaum, SL (2007) "Osteoclasts: What Do They Do and How Do They Do It?" *The American Journal of Pathology*. 170(2): 427-35.

vanCorven EJ, van Rijswijk A, Jalink K, van der Bend RL, van Blitterswijk WJ, Moolenaar WH. (1992) "Mitogenic Action of Lysophosphatidic Acid on Fibroblasts, Dependence on Acyl-Chain Length and Inhibition by Suramin." *Biochemistry Journal*. 281(Pt 1): 163-9.

Zeng Q, Lagunoff D, Masaracchia R, Goeckeler Z, Cote G, Wysolmerski R. (2000) "Endothelial Cell retraction is Induced by PAK2monophosphorylation of Myosin II." *Journal of Cell Science*. 113(3): 471-82.

Zou W, Teitelbaum SL. (2010) "Integrins, Growth Factors, and the Osteoclast Cytoskeleton." *Annals of the New York Academy of Science*. 1192 (1): 27- 31.

Figure 1

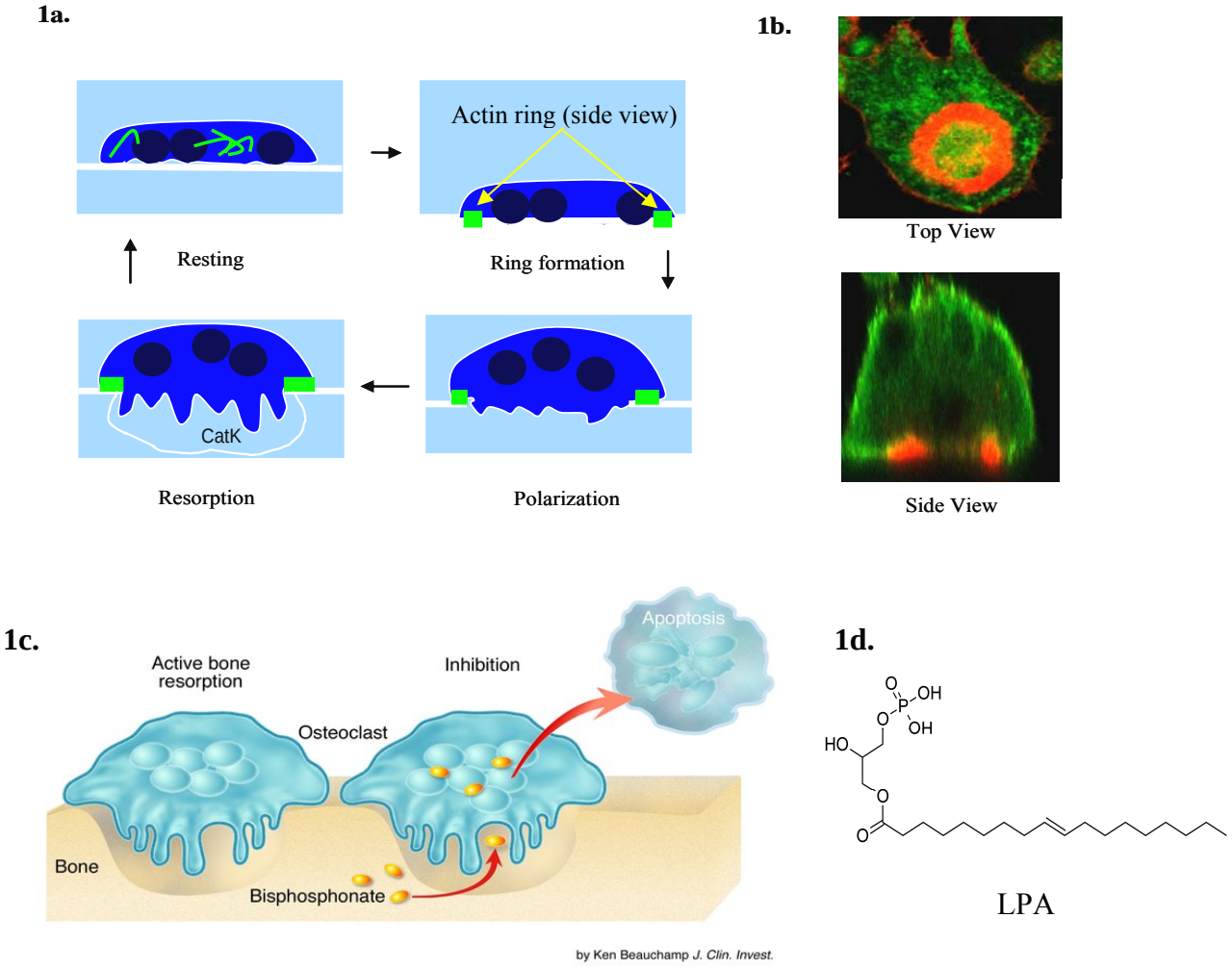
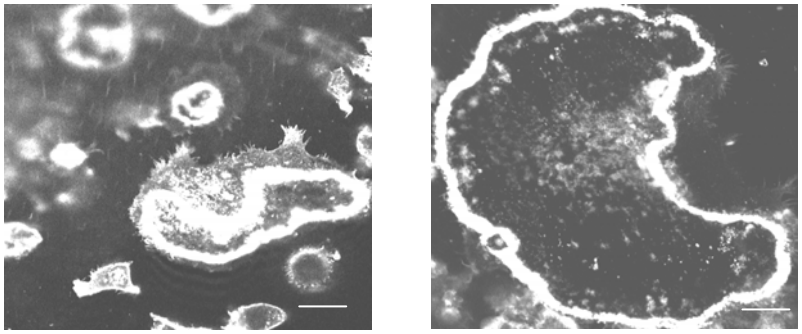


Figure 1: Actin sealing zone formation is essential to bone resorption by osteoclasts. (**1a).** As the osteoclast progresses from the resting state (top left) through to the resorption phase, the osteoclast polarizes and the actin sealing zone forms. The release of Cathepsin K (Cat K) and HCl result in degradation of the bone matrix below the sealing zone area. (**1b).** *Top*: Aerial view of osteoclast. The red coloring indicates the actin sealing zone. *Bottom:* Lateral view of the osteoclast. **(1c).** Bisphosphonates induce apoptosis in osteoclasts. When administered, bisphosphonates adhere to the bone surface and are eliminated from the circulation, leaving most other somatic cells unaffected. However, when osteoclasts resorb bone, they uptake the bisphosphonates along with the bone matrix. The drugs are designed to induce apoptosis of the osteoclasts via an intracellular mechanism. (Beauchamp 2002) (**1d**). The chemical structure of lysophosphatidic acid (LPA).

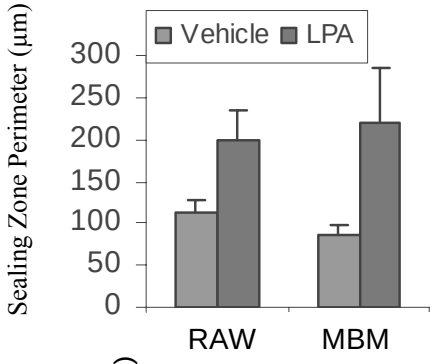
Figure 2

2a.

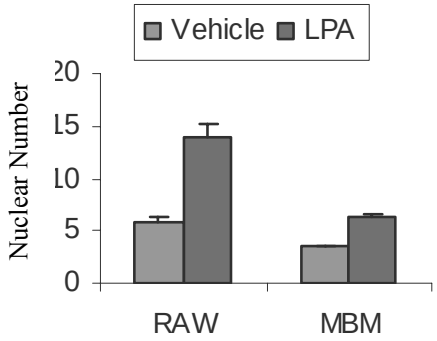


Untreated Vehicle Control **LPA**

2b.



2c.



2d.

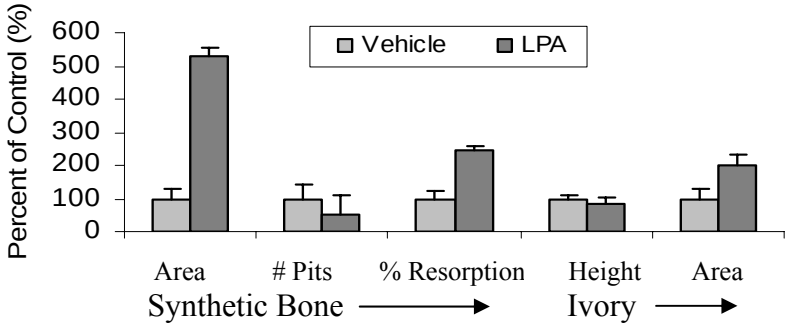
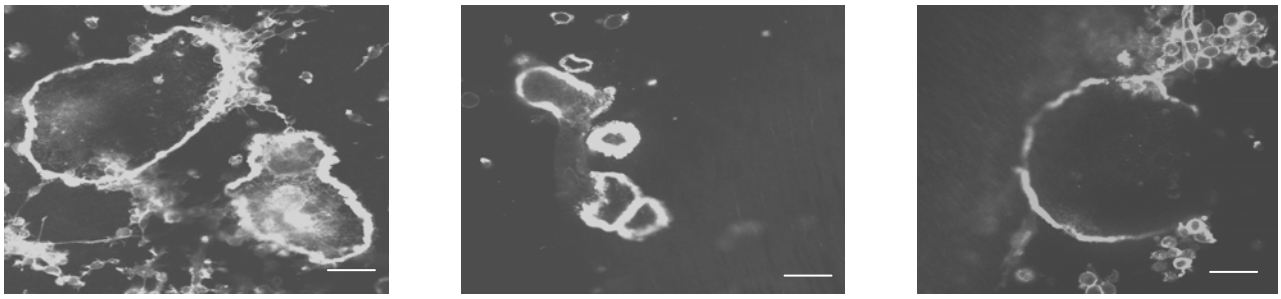


Figure 2: **LPA increases actin sealing zone size. (2a).** The top left photo shows the actin sealing zone of an untreated osteoclast in vehicle solution alone, while the photo on the right illustrates the sealing zone phenotype that results when osteoclasts are exposed to lysophosphatidic acid (LPA). The actin has been fluorescently stained, and can be distinguished as a bold ring structure. Scale bar = 10 µm (**2b).** The graph on the left indicates expansion of the actin sealing zone in osteoclasts treated with LPA, relative to the vehicle control cells. **(2c).** The rightmost graph indicates that the nuclear number also increases upon addition of LPA in both subsets of RAW and MBM cells, trending with the larger sealing zone perimeter of the LPA-exposed osteoclasts. (**2d**). On a synthetic bone medium, LPA addition results in a significant increase in the individual pit area resorbed, and in the total percent of the bone resorbed. On an ivory medium, LPA addition results in a significant increase in the individual pit area resorbed, but shows no significant increase in the height of the resorption pit relative to the untreated vehicle control.

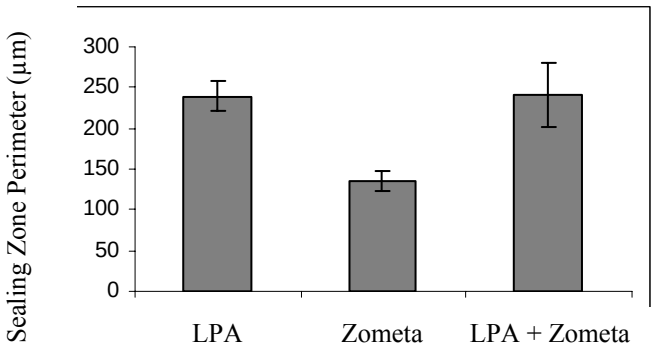
Figure 3

3a.



LPA alone **Zometa alone** **LPA + Zometa**

3b.



3c.

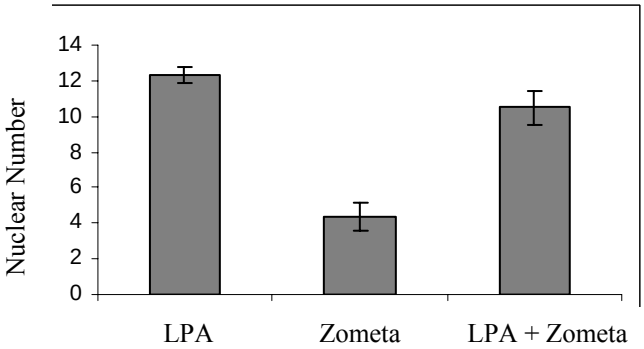
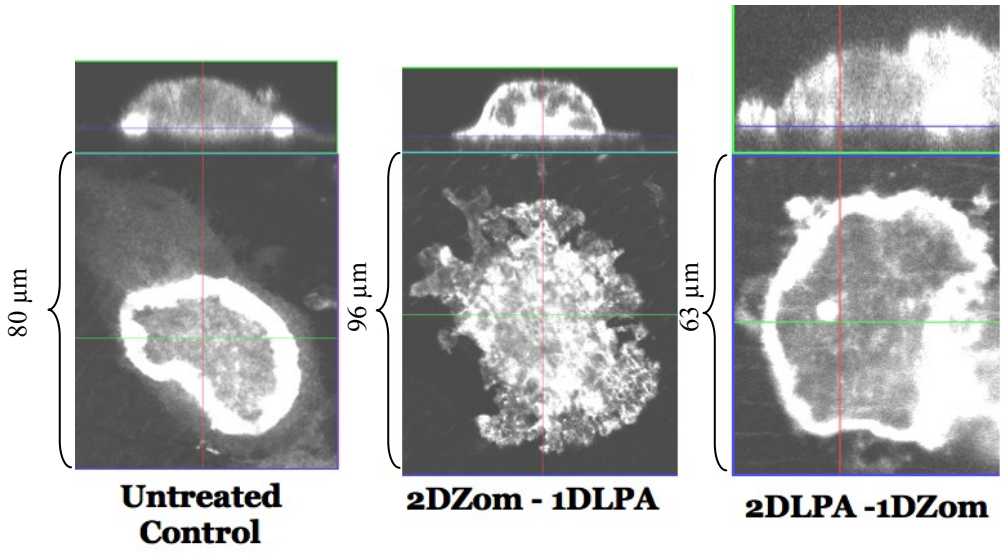


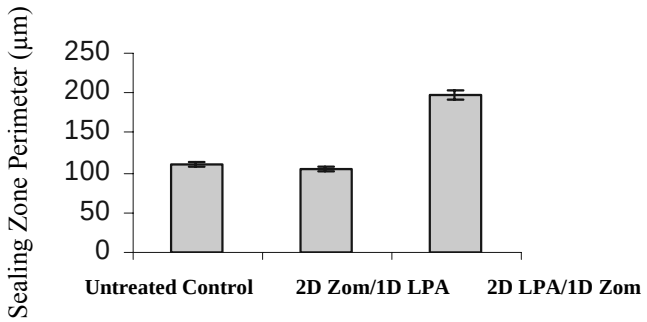
Figure 3: LPA and Zometa have contrary effects. (3a). This figure illustrates the relative actin sealing zone sizes (bold ring structures), under conditions of LPA alone (left), Zometa only (middle) and LPA and Zometa added simultaneously (right). Scale bar = 10 µm. **(3b).** This graph shows that adding LPA and Zometa to cells simultaneously produces actin sealing zones with a larger perimeter, comparable to the LPA-exposed phenotype. **(3c).** Adding LPA and Zometa to cells results in an increased nuclear number. This nuclear number is statistically similar to the number of nuclei present in osteoclasts exposed to LPA alone.

Figure 4

4a.



4b.



4c.

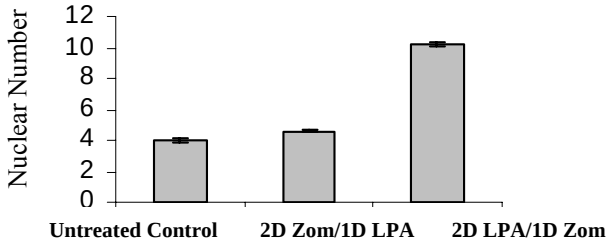
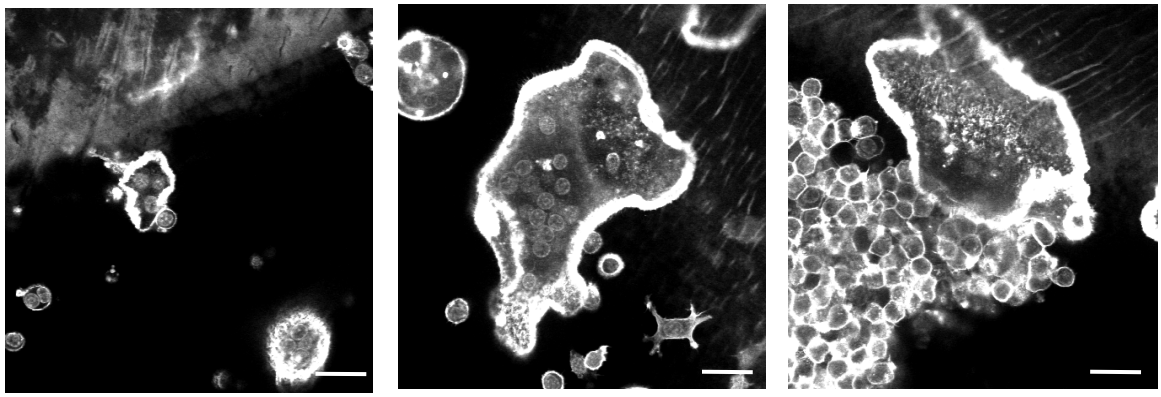


Figure 4: Pre-treatment with LPA makes Zometa ineffective. (4a) A time assay of osteoclast exposure to LPA and Zometa at different time points reveals that applying LPA to cells 24 hours prior to adding Zometa to the culture media makes Zometa ineffective. Compared to the untreated control, cells treated with Zometa prior to LPA treatment (2D Zom-1D LPA) exhibited decreased actin ring size (or no ring formed at all), while cells treated with LPA first (2D LPA-1D Zom) displayed an actin sealing zone measuring approximately double the size of sealing zone of the untreated control. **(4b).** The addition of LPA to osteoclasts 24 hours before Zometa results in a two-fold increase in the actin sealing zone. **(4c)**. The addition of LPA to osteoclasts 24 hours before Zometa results in a higher nuclear number, corresponding to the increased size of the actin ring.

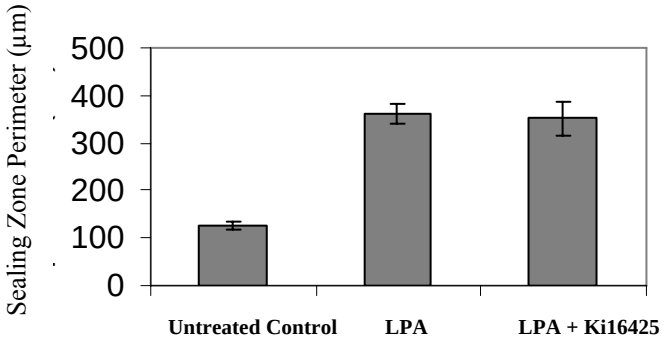
Figure 5

5a.



Untreated Control **LPA** **LPA + Ki 16425 Inhibitor**

5b.



5c.

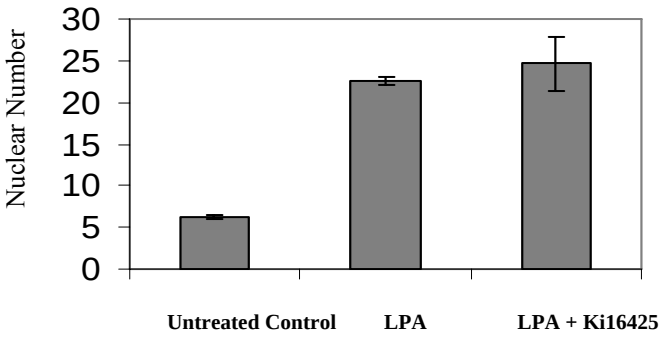
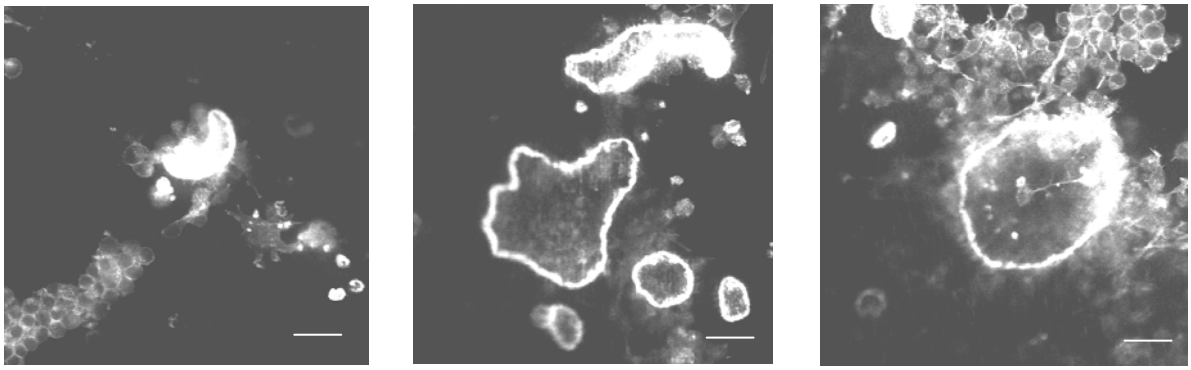


Figure 5: LPA Not Inhibited by LPAR1,3 Antagonist Ki16425. (5a). The Ki16425 inhibitor, which inhibits LPAR1 and LPAR3 gene products, produces the same actin ring phenotype in cells as found with LPA. Scale bar = 10µm. **(5b)** and **(5c).** Osteoclasts exposed to both LPA and LPA + Ki16425 inhibitor result in an expanded actin ring perimeter, as well as an increased number of nuclei. Since LPAR1 and LPAR3 were inhibited and the LPA-regulated sealing zone expansion was not hindered, it is not likely that LPAR1 or LPAR3 are significant mediators in the ring expansion mechanism.

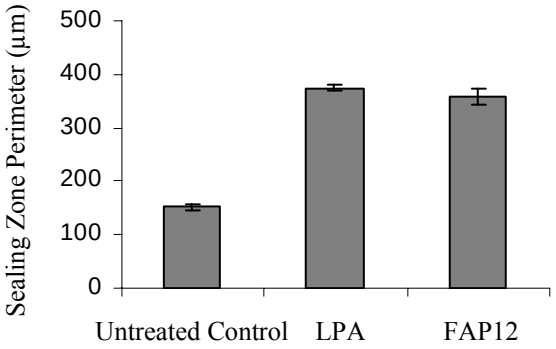
Figure 6

6a.

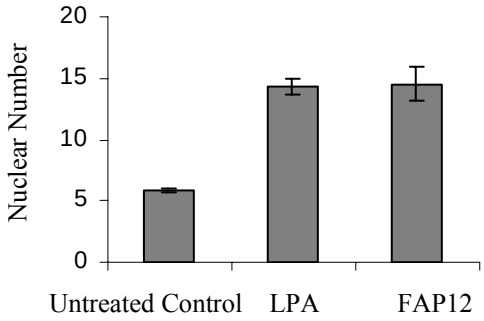


Vehicle Control **LPA** **FAP12 agonist**

6b.



6c.



6d.

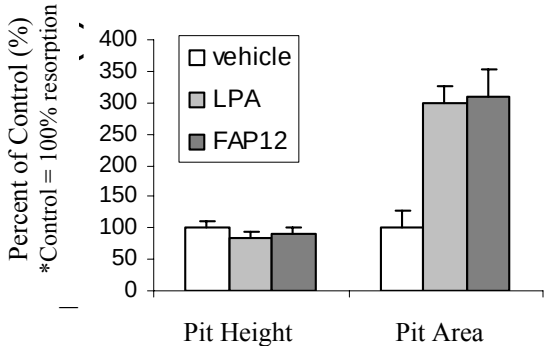
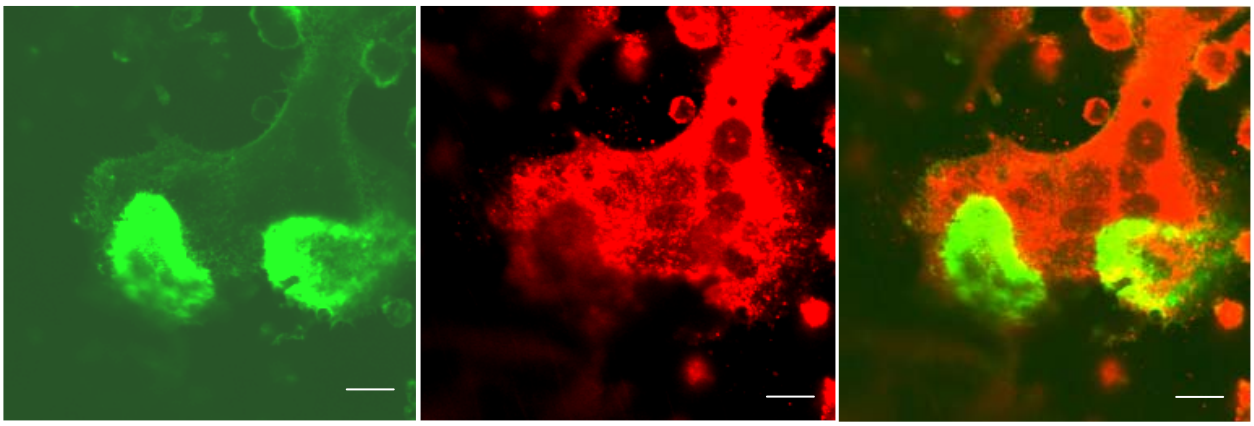


Figure 6: LPAR2 Agonist FAP12 Mimics LPA Effects. (6a). The FAP12 agonist, which is specific to LPAR2, produces the same actin ring phenotype in cells as found with LPA. Both LPA and FAP12 result in an expanded actin ring relative to the untreated control. Scale Bar = 10µm **(6b)** and **(6c).** Osteoclasts exposed to both LPA and FAP12 agonist result in an expanded actin ring perimeter, as well as an increased number of nuclei. The expanded actin sealing zone observed can be correlated with activation of LPAR2 as a result of this experiment. (**6d**). The LPA agonist FAP12 mimics LPA resorption patterns; addition of LPA or FAP12 to cells results in an increased resorption pit area relative to the untreated control.

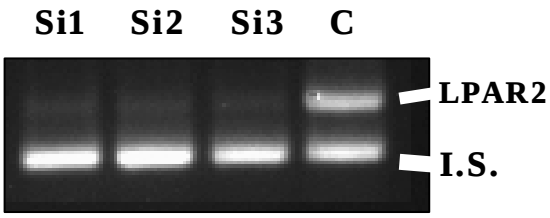
Figure 7

7a.



F-Actin Sealing Zone **LPAR2** **Both**

7b.



7c.

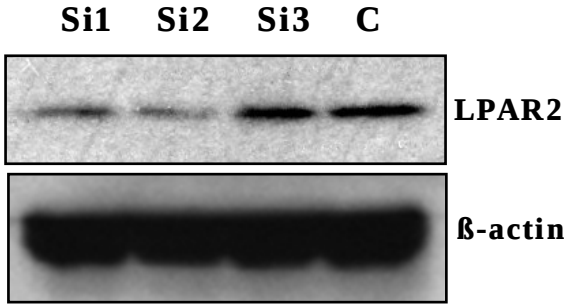
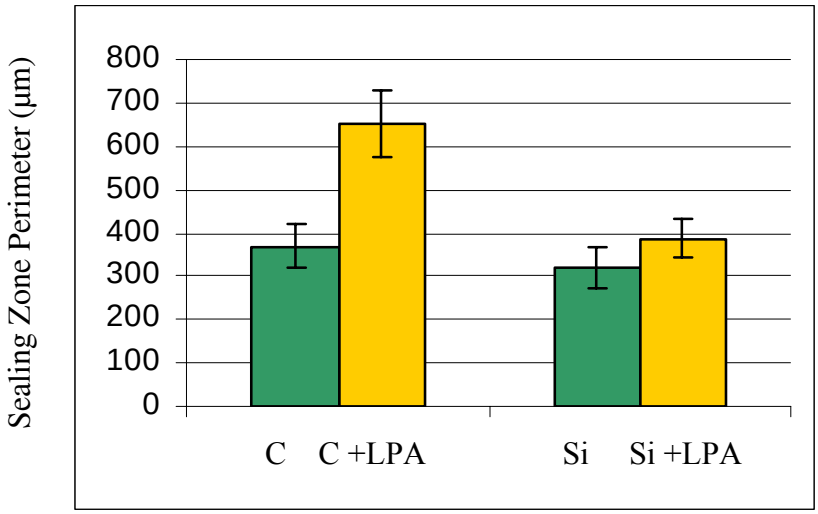


Figure 7: siRNA Suppression of LPAR2 diminishes LPA Effects
(7a). Osteoclasts were stained with LPAR2-specific antibodies, which fluoresced red (middle). Actin was stained with Phalloidin and fluoresced green (left). The rightmost image confirms that LPAR2 is located throughout the osteoclast cell membrane, yet is not present in the actin ring structure. Scale bar = 10 µm. **(7b).** A competitive reverse-transcriptase PCR (RT-PCR) of the three LPAR2-specifc siRNAs shows that the siRNAs have effectively induced degradation of the cellular LPAR2 RNA products. The relatively darker bands indicate that a higher concentration of each of the three siRNAs was amplified in comparison to the LPAR2 gene product. The control lane indicates that the initial amounts of internal standard (I.S.) and the LPAR2 gene product were approximately equal in the PCR mixture. Such a control attributes any band discrepancies to the I.S. being selectively amplified during PCR, as a result of the knocked-down LPAR2 gene RNAs. **(7c).** Western blot assays indicate that all three siRNAs knocked down the production of the LPAR2 protein. siRNA 1 and siRNA 2 were most effective at suppressing LPAR2 expression. A relatively constant amount of ß-actin protein, present in all cells, ensures that the wells of the agarose gel were loaded consistently, and any change in the intensity of the protein bands can be explained by hindered protein production alone.

Figure 8

The Effect of LPA on LPAR2 siRNAs

(8a)



(8b)

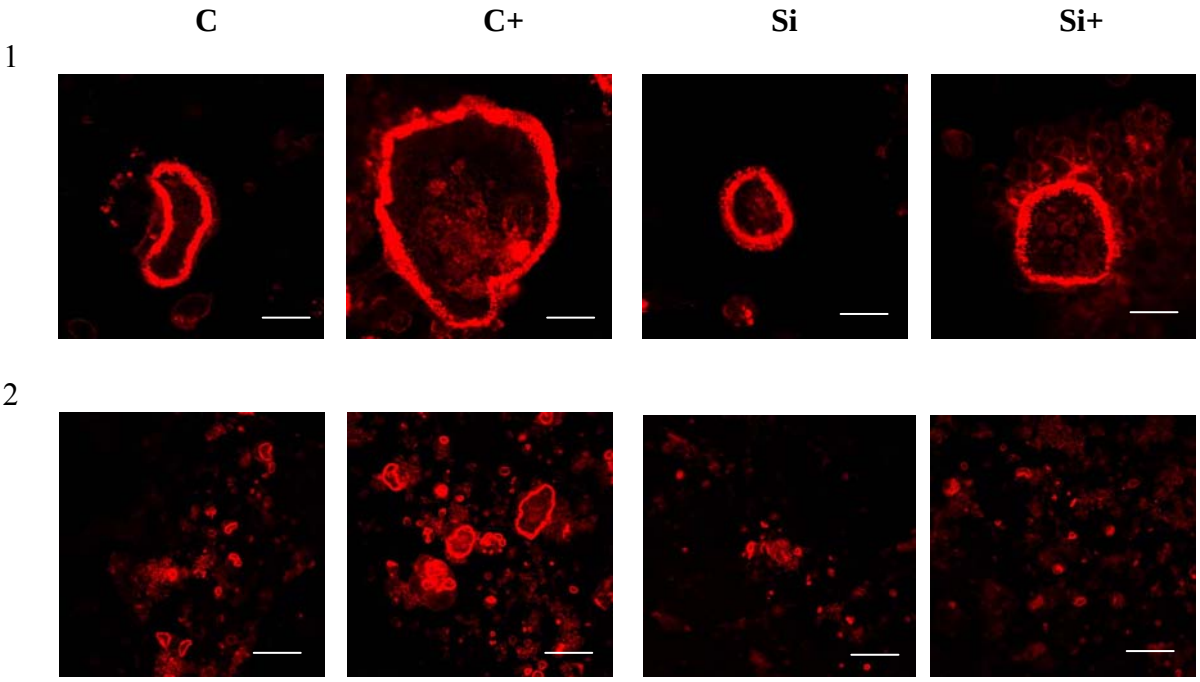


Figure 8: LPA is Responsible for Formation of Expanded Actin Sealing Zone. (8a). Osteoclasts still exhibit the expanded actin ring phenotype, even when transfected with a control (non-specific) siRNA. This observation confirms that the siRNAs are not involved in increasing the sealing zone perimeter. LPA interaction with LPAR2 is crucial to sealing zone expansion, since the cells transfected with LPAR2-specific siRNAs do not exhibit the larger sealing zone, even in the presence of LPA. **(8b).** Epifluorescent microscopy images compare the effects of control and LPAR2-specific siRNA transfections on actin ring structures. Row 1 illustrates images at higher magnification (80x, scale bar = 10µm), while Row 2 shows a wider field at a lower magnification (20x, scale bar = 56 µm).

ABBREVIATIONS SUMMARY

CatK – Cathepsin K

DNA – Deoxyribonucleic Acid

DMEM – Dulbecco's Modified Eagle Medium

LPA – Lysophosphatidic Acid

LPAR– Lysophosphatidic Acid Receptor

MBM – Mouse Bone Marrow

RANKL – Receptor Activator for Nuclear Factor κ B Ligand

RNA – Ribonucleic Acid

RT-PCR – Reverse Transcription – Polymerase Chain Reaction

siRNA – small interfering Ribonucleic Acid